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[1*]A. Baubekova, [2]Sh. Akhmetsadykova, [1,2]A. Kondybayev,
[3]B. Faye, [1]G. Konuspayeva

[1]Al-Farabi Kazakh National University, Almaty, Kazakhstan
[2] Research and Production Enterprise "Antigen" Co. Ltd, Almaty region, Kazakhstan
[3]UMR Selmet, Cirad, Montpellier, France
[*]e-mail: Almagul.Baubekova@kaznu.kz

Volatile organic compounds profile of *Lactobacillus casei* and *Streptococcus thermophilus* in fermented mare milk of Kazakhstan

Abstract: Fermented milk is a common beverage in many countries, but its flavor is highly variable from one region to another. The fermented mare milk, common beverage in Central Asia, has a typical flavor of *koumiss*, but those flavors were never described using aroma compound determination. In the present study, six lactic acid bacteria were identified in local *koumiss*. Due to the apparent technological advantage of *Lactobacillus casei* and *Streptococcus thermophilus* (notably their acidification speed), the main objective of this study was to compare their volatile compound profiles. 35 aromatic compounds were detected. To compare the aroma profiles of each strain, the ratio proportion strain/control was calculated for each strain and for each aroma showing significantly different profiles with predominance of 2-methyl-1-propanol and 2-undecanol in milk fermented with *L. casei*, and of 2,3-pentanedione in milk fermented with *S. thermophilus*. *L. casei* strain produced more aroma and its profile was significantly (P>0.03) different from *S. thermophilus* profile.
Key words: mare milk, *koumiss*, *Lactobacillus casei, Streptococcus thermophilus*, aromatic compounds.

Introduction

Fermented milk is a common beverage in many countries, but their taste is highly variable from one region to another [1]. Flavor development mainly depends on the microflora, composed of lactic bacteria and yeasts [2]. The main biodiversity of taste in fermented products as wine is due to "terroir", which depends on different factors: local microflora, traditional tools and dishes used, climatic conditions (temperatures, humidity), etc. The characterization of microflora from fermented dairy products originated from non-cattle milk are few investigated especially, there is few data regarding the microflora biodiversity in fermented mare milk [3]. Traditionally, mare milk is produced and consumed in Central Asian countries, such as Kazakhstan, Mongolia, Kyrgyzstan, Uzbekistan, Russia, China (western provinces) [4].

In other countries, mare milk is consumed under milk form, essentially for newborn babies in France, in Germany etc. The main strains isolated in fermented mare milk (in international data called koumiss, and phonetically in Kazakh – *koumiss*), are lactic bacteria such as *Lactobacillus* (*L.*) *casei, L. helveticus, L. plantarum, L. fermentum, L. acidophilus, Leuconostoc mesenteroides, Lactococcus lactis, Streptococcus* (*S.*) *thermophilus, Enterococcus faecalis, Enterococcus durans, Enterococcus casseliflavus;* and yeasts, such as *Kazachstania unispora, Saccharomyces cerevisiae, Torulopsis koumis* [3; 5-11].

The characterization of aroma production by microflora was mainly achieved in cheese, notably after ripening [12-15]. The fermented mare milk has a typical flavor of *koumiss* [16]. However, those flavors are generally described as organoleptic properties, only with words, such as acid, milky, creamy, sweet and some other. Traditionally produced on-farm by mixing fresh milk with already fermented milk; the organoleptic qualities of *koumiss* are highly variable. Face to industrialization of the process, there is necessity to standardize the production. For that, the characterization of aroma by selected microflora is a way to propose standardized *koumiss* to the consumers. At our knowledge, the aroma description of fermented mare milk was never achieved.

As the target of our strain selection was to prepare starters for fermentation process at industrial

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level [17]. Lactic acid bacteria (LAB) strains were tested for their acidification speed and their survival capacity in selective milieu for industrial use, especially acid-resistance and growth speed in bioreactor [18]. Finally, the main objective of the present study was to compare the volatile compound profile of mare milk fermented separately by the selected strains among the natural lactic bacteria.

Materials and methods

Analyzed samples and microflora isolation. One sample of *koumiss* with typical pleasant taste was collected in Almaty region, Saryzhailau dairy plant for isolation of natural microflora. The LABs were isolated on MRS and M17 media at the conditions described *infra* in Table 1.

Table 1 – Lactic acid bacteria strains isolated from koumiss in Almaty region

No.	Microorganism code	Growth medium	Growth conditions in broth	Growth conditions on agar
1	*K5*	*MRS*	37°C/AE, 24 h	37°C/AE, 24 h
2	*K7*	*MRS*	37°C/AE, 24 h	37°C/AE, 24 h
3	*K5.C*	*MRS*	37°C/AE, 24 h	37°C/AE, 24 h
4	*K14*	*MRS*	37°C/AE, 24 h	37°C/AE, 24 h
5	*K12*	M17	42°C/AE, 24 h	42°C/AE, 48 h
6	*K17*	M17	42°C/AE, 48 h	42°C/AE, 48 h

Identification of strains. To identify the strains, polymorphism in the DNA fragment of ribosomal 16S was used according to the methods of Ampe and Leesing [19; 20]. The purity and quantity of extracted DNA was verified by measuring the absorbance (Biospec-Nano, Japan), and by electrophoresis and loaded into 0.8% agarose gel in 1x TAE buffer (40 mM Tris-HCl, pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2-EDTA, Eppendorff, Hamburg, Germany) with a molecular weight ladder 1kb (Supercoiled DNA Ladder, Invitrogen, USA). After running at 100 V for 40 min, the gels were stained for 30 min in an ethidium bromide solution (50 μg mL/1; Promega), rinsed for 10 min in distilled water, then observed and photographed on a UV transilluminator.

PCR amplification of extracted DNA. The V3 variable region of bacterial 16S rDNA was amplified using Seqfor1 (5'-GGA AAC AGA TGC TAA TAC CG-3', Sigma) and seqrev 1 (5'-GCT GCT GGC ACG TAG TTA-3', Sigma) primers.

PCR amplifying procedure was as follows: 3 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 46°C, 1 min at 72°C and then 5 min at 72°C. It was carried out on the automatic thermal cycler (PTC-100 Peltier Thermal Cycler). Isolation and purification of PCR products was carried out using the Wizard PCR Preps (Promega, USA) kit, as recommended by the manufacturer. The sequencing of purified products was performed by GATC Biotech (Germany). Raw sequences data were edited using Sequence Scanner software and compared to the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) and the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp). Sequences having a percentage of identity of 97% or greater were considered to belong to the same species [21; 22].

Acidification tests and growth kinetic of the identified LABs. The process of lactic acid bacteria fermentation was evaluated using Cinac. The Cinac system (INRA, France), allows continuous monitoring of the activity of acid-enzymatic fermentation by simultaneously observing changes in pH and temperature in several samples. LAB was inoculated into ultra-sterilized cow milk (2.5% fat, UHT, bio, Casino, France) in an amount of 2% and cultured at optimal temperature conditions: 42±1°C for thermophilic *Streptococci*, 37±1°C for *Lactobacilli.*

All tests were carried out in duplicate. After 24h of incubation, 2.5 mL aliquots of the samples were placed in vials (PerkinElmer, 22 mL) with polytetrafluoroethylene (PTFE)/silicone septa. Samples were stored at -80°C until analysis of volatile compounds.

Analyses of volatile compounds (VOC). Samples were analyzed by the team from UMR 1253 STLO (INRA-Rennes, France). Aliquots (2.5 g) of cultures were placed in 22 mL PerkinElmer vials with polytetrafluorethylene/silicone septa. Vials were stored at -80°C until analysis of volatile compounds. Samples were randomized in the sample list.

Extraction of volatile compounds. A Perkin Elmer Turbomatrix HS-40 trap automatic headspace sampler with trap enrichment was used to extract

volatiles according to Pogacic et al, 2015 [15]. The principle of the HS-trap method has been previously described in detail [23; 24]. It includes several steps: equilibration, pressurization, trap load, trap dry-purge, trap desorption and trap hold. Conditions were as follows. Samples were warmed for 15 min at 65°C (equilibration). The vials were then pressurized for 1 min at 207 kPa with the carrier gas (helium), by introducing a needle through the septum. A Tenax™ trap at 35°C was then loaded for 2.3 min by allowing the pressure to fall through the trap, permitting the adsorption and the concentration of the analytes of the headspace on the trap. The trap load was repeated twice for each vial trap. The adsorbed water was then removed by purging helium through the trap (dry purge: 3 min). The trap was heated at 250°C for 0.1 min and back flushed at 89 kPa, leading to desorption of the analytes, which were then transferred to the GC through a transfer line maintained at 150°C, with an injection time of 0.6 min. The trap was held at 250°C for 5 min.

Analysis of volatile compounds. Volatiles were analyzed using a Clarus 680 gas chromatograph coupled to a Clarus 600T quadrupole mass spectrometer (Perkin Elmer, Courtaboeuf, France). They were separated on a Stabilwax-MS capillary column (30 m x 0.25 mm x 0.25 µm; Restek, France), with helium as the mobile phase. The initial temperature of the oven, 30°C, was maintained for 10 min. The increase of temperature was performed at a rate of 5°C/min up to 230°C. The mass spectrometer was operated in the scan mode (scan time 0.3 s, interscan delay 0.03 s) within a mass range of m/z 29 to 206. Ionization was done by electronic impact at 70 eV. Standards were regularly injected to verify the absence of instrumental drift of the GC-MS system. Blank samples (boiled deionized water) were also injected to check the absence of carry-over. Volatile compounds were identified by comparison of mass spectra and retention times with those of authentic standards purchased from Sigma-Aldrich (St-Quentin-Fallavier, France), on the basis of their retention index and mass spectral data from the NIST 2008 Mass Spectral Library (Scientific Instrument Services, Ringoes, NJ, USA). Some compounds were tentatively identified on the basis of mass spectral data only when data on retention indices were not available.

Data processing. Data pre-processing was performed using PerkinElmer Turbomass software, version 5.4.2.1617. The GC-MS raw data files were converted to netCDF format with Data Bridge (Perkin Elmer, Waltham, Massachusetts, USA) for further analysis. GC-MS data were processed by converting the raw data to time- and mass-aligned chromatographic peaks areas using the open source XCMS package implemented with the R statistical language [25]. The full width at half maximum was set to 5, the group band-width to 3, and the other parameters were those by default.

Statistical data analysis. Each strain was analyzed in triplicate and the mean was retained as convenient value after verification of a low variation coefficient. This coefficient for triplicates was all the times below 1%. It was on average 0.011% (range 0.001-0.058% according to compound) for control sample, 0.026% (0.001-0.107) for *L. casei* sample, and 0.018% (0.002-0.063) for *S. thermophilus* sample. The aroma profiles were compared graphically. To facilitate the comparison, the quantitative values were presented as percentage of total aroma in the results table, and transformed in log10 values for giving a better reading of the profiles' graphic. Due to the lack of homogeneity of the variance, a non-parametric test (Mann-Whitney test) was applied to compare the two entire profiles by using the software XLstat (Addinsoft©).

Results and discussion

LABs were identified by NCBI and RDP data bases with 100% confidence with Seqfor1 and Seqrev1 system of primers. Finally, six LAB strains were isolated and identified belonging to genus *Lactococcus*, *Leuconostoc*, *Lactobacillus* and *Streptococcus* (Table 2).

Table 2 – Identification of lactic acid bacteria strains isolated from koumiss

No.	Microorganism code	Identified microorganism
1	*K5*	*Lactococcus lactis*
2	*K7*	*Leuconostoc lactis*
3	*K5.C*	*Leuconostoc lactis*
4	*K14*	*Lactobacillus casei*
5	*K12*	*Streptococcus thermophilus*
6	*K17*	*Streptococcus thermophilus*

After 18 hours of incubation during acidification tests, pH of fermented milk with specific strains decreased from 6.5 to 4.14 for *Lactobacillus casei K14* (*L. casei*) and to 5.22 for *Streptococcus thermophilus K12* (*S. thermophilus*). The acidification speed of the

other strains was slower. Moreover, one of the most common points regarding *koumiss* microflora in the literature is the common presence *of L. casei* and *S. thermophilus*. Those species have high technological interest and could play also therapeutic effect on liver damages [26]. So, finally, those two LAB strains were selected for the VOC analysis.

As the whole, 35 volatile aroma compounds produced by studied strains and control sample of milk were identified (Figure 1).

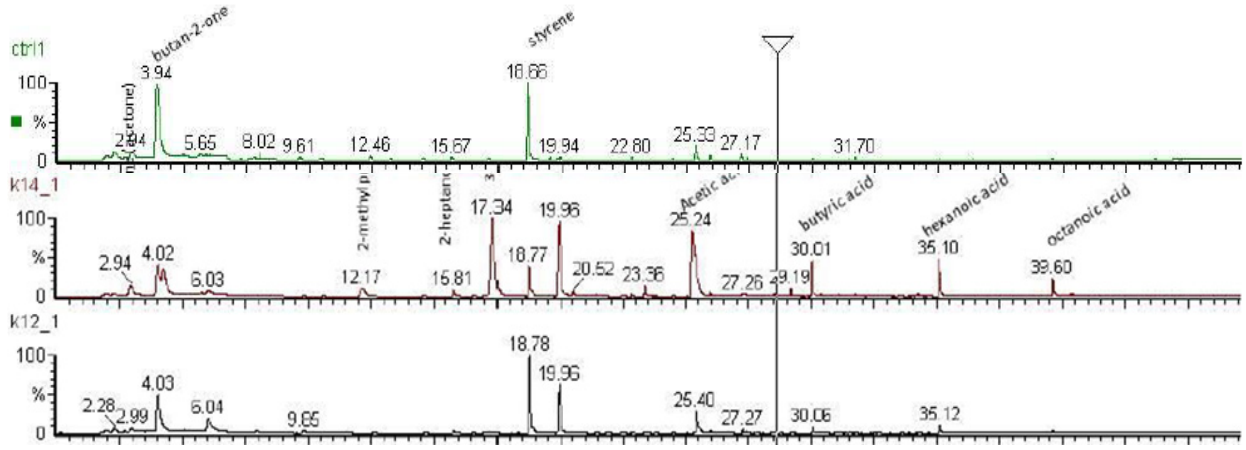

Figure 1 – Chromatographic profile of each samples of strains

The main aroma compounds among the 35 detected ones (representing more than 3.5% in percentage, i.e. above the mean of percentage of 100/35) found in control sample were styrene and 2-butanone. In sample of fermented milk inoculated with *L. casei*, the main compounds were methylbenzene, 2,3-butanedione, styrene, 2 butanone, 3-hydroxy, and 2-butanone. In milk fermented with *S. thermophilus*, they were hexanoic acid, 3-methylbutanal, butanoic acid, 2-butanone, 1-butanol, 3-methyl, 2 butanone, 3-hydroxy and acetic acid (Table 2).

Table 2 – Volatile aroma proportion (the total of column is 100%) of milk fermented by each strain and control (without fermentation, milk only)

Name	Ion	TR	CAS number	Identification database	Control	*L. casei*	*S. thermophilus*
2-undecanol	97	32.33	1653-30-1	RI, DB	0.0002	0.0006	0.0152
2-methyl-1-propanol	74	12	78-83-1	S, RI, DB	0.0007	0.0005	0.2108
2,3-pentanedione	100	9.78	600-14-6	RI, DB	0.0014	0.3684	0.0004
propionic acid, 2-methyl	73	28.5	79-31-2	S, RI, DB	0.0033	0.0067	0.1613
2-tridecanone	71	34.33	593-08-8	RI, DB	0.0050	0.0187	0.0515
2-propanone, 1-hydroxy	74	20.5	116-09-6	RI, DB	0.0061	0.0099	0.1230
propionic acid	74	27.76	79-09-4	S, RI, DB	0.0103	0.0350	0.1043
2-buten-1-ol, 3-methyl-, acetate	67	21.43	1191-16-8	RI, DB	0.0128	0.0308	0.1222
2-undecanone	58	29.18	112-12-9	RI, DB	0.0182	0.1264	0.6356
2-butenal-3-methyl	84	16.55	107-86-8	RI, DB	0.0246	0.0226	0.1718

Continuation of Table 3

Name	Ion	TR	CAS number	Identification database	Control	*L. casei*	*S. thermophilus*
hexanol	56	22.47	111-27-3	RI, DB	0.0280	0.1299	0.0764
butanoic acid, 3-methyl	60	31.02	503-74-2	S, RI, DB	0.0357	0.0584	0.2868
1-butanol, 3-methyl	55	17.3	123-51-3	S, RI, DB	0.0415	0.1065	15.7518
hexanal	82	10.65	66-25-1	RI, DB	0.0663	0.0022	0.0005
p-xylene	106	13.07	106-42-3	S, RI, DB	0.0886	0.0091	0.0022
octanoic acid	60	39.58	124-07-2	S, DB	0.0889	0.7855	0.9837
2-nonanone	58	23.33	821-55-6	S, RI, DB	0.1814	0.2485	1.2712
2-pentanone	86	5.75	107-87-9	RI, DB	0.1985	0.1197	0.1913
hexanoic acid	60	35.08	142-62-1	S, DB	0.2136	1.6628	4.1169
acetic acid	60	25.4	64-19-7	S, RI, DB	0.2965	2.6020	22.7597
dimethyl sulfone	79	36.42	67-71-0	DB	0.3180	0.1723	0.1991
2 butanone, 3-hydroxy	45	19.93	513-86-0	S, RI, DB	0.3460	25.3227	18.8271
3-methylbutanal	58	4.2	590-88-3	S, RI, DB	0.3549	0.1514	4.4287
2,3-butanedione	86	6	431-03-8	S, RI, DB	0.3618	9.4461	1.1770
butanoic acid	60	30	107-92-6	S, RI, DB	0.4051	1.8169	7.8582
methylbenzene	92	7.33	108-88-3	RI, DB	0.5098	3.5170	0.1331
1-butanol	56	14.6	71-36-3	RI, DB	0.5405	0.0566	0.0386
2-heptanone	58	15.78	110-43-0	S, RI, DB	0.7164	0.4933	0.7284
benzaldehyde	105	27.23	100-52-7	S, RI, DB	0.7377	0.4935	0.1209
ethylbenzene	91	12.63	100-41-4	RI, DB	1.4873	0.5172	0.1002
acetone	58	2.98	67-64-1	RI, DB	2.5985	1.4827	0.5605
styrene	104	18.75	100-42-5	RI, DB	15.1948	19.8396	3.4508
2-butanone	43	4	78-93-3	S, RI, DB	75.1078	30.3467	15.3409
Total					100%	100%	100%

Note: S – Retention time and mass spectrum from standard, RI – Retention index, DB – Mass spectral data

To compare the aroma profiles of each strain, the ratio proportion strain/control was calculated for each strain and for each aroma (Figure 2).

L. casei strain from *koumiss* produced more aroma compounds and its profile was significantly ($P>0.03$) different of *S. thermophilus* profile. 3-methylbutanal, 2-pentanone and dimethyl sulfone were produced mainly by *L. casei* and not by *S. thermophilus*. At reverse, methylbenzene, styrene, benzaldehyde and 2,3-pentanedione were mainly produced by *S.thermophilus* and few by *L. casei*. Capacity of lactic acid bacteria to produce aroma in *koumiss* was similar to that described by Helinck et al. [27].

As the production of volatiles is highly strain-dependent (both qualitatively and quantitatively) and is depending also on the conditions, it is difficult to compare our results with those of the literature. The comparison to the data obtained using other strains, on other dairy products such as cheese, and extracted with other GC-MS methods is quite debatable. Moreover, no data regarding VOCs in mare milk was available in the literature. In one reference regarding Cheddar cheese where *L. casei* is dominating LAB, the main aromas were nonanoic, undecanoic, heptanoic and myristoleic acid and their ethers [14]. Branched aldehydes, such as 3-methyl butanal are important flavor compounds in many food products, fermented and non-fermented (heat-treated) and are products of *S. thermophilus* strains [27; 28]. Such aroma pattern appeared close to that isolated from our *koumiss* samples.

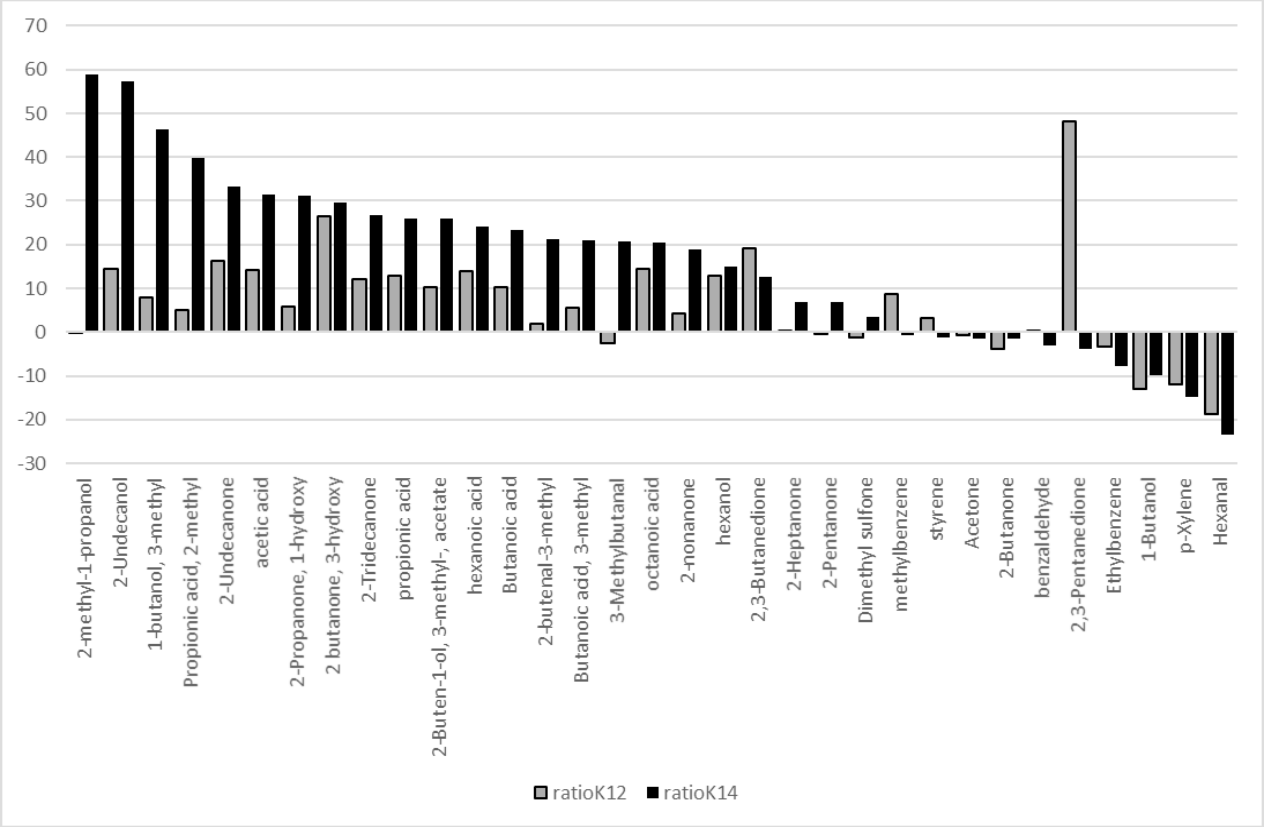

Figure 2 –Ratio of strain aroma compounds /control aroma compounds for *L.casei* (in black) and *S.thermophilus* (in grey). The aroma compounds are in decreasing order for *L. casei*

Obviously, the proportion of each aroma can be lower even if the absolute amount increases, and the total was the sum of the abundance of one selected fragment for each compound and not the sum of total current intensity (TIC) value. Moreover, the flavour-impacting compounds are rarely the dominant ones, and that the method of extraction markedly influence the nature and ratio of the compounds extracted. The present results aimed only to describe the global "aroma profile" of two strains expected to be used in specific starters. Further investigations regarding the sensory analysis should be necessary to assess potential relationship between felt flavor and aroma profile.

Conclusion

The present characterization of *L. casei* and *S. thermophilus* from *koumiss* samples was new and until now no data was available. 35 aroma compounds were detected in fermented and control samples. *L. casei* showed greater aroma producing capacity. Due to the place of traditional fermented dairy products in Central Asia, the description of aroma profiles of *koumiss* could be an important step in the modernization of this specific sector. It is a good potential for improving the technology of production at industrial level. However, it would be necessary to complete such analysis by using several strains of the same species grown under the same conditions and using the same method.

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